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Applicants: Miri Seiberg, et al.

Serial No.: 09/698,454

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Examiner: M. Lamm

For: SOY DEPIGMENTING AND SKIN CARE COMPOSITIONS

DECLARATION OF JONATHAN D. MILLER

I, Jonathan D. Miller, hereby declare:

1. I am a named inventor of U.S. Patent Application Serial No. 09/698,454, filed October 27, 2000. I am employed by Johnson & Johnson Consumer Companies, Inc. in the position of Senior Scientist and Go-to-Market Leader. I received a Bachelor of Science degree in Chemical Engineering from Cornell College of Engineering in 1999. After receiving my Bachelor of Science degree I became employed by Johnson & Johnson in 1999. Since then, my duties have centered on the development of new skin care products in the Department of Research and Development

2. I participated in developing compositions containing non-denatured soy products that retained their biological activity and were physically and chemically stable. During the course of developing such soy-containing products, at my direction, studies were conducted to determine the physical and chemical stability of chemical compositions containing non-denatured soy.

3. At my direction, Compositions A and B were formulated. Compositions A and B contained the following ingredients:

Composition A:

Excipient	Concentration. %w/w
Deionized Water	70.58
Glcyerin	3.00
Preservative	0.73
Ascorbyl glucoside	2.00
Panthenol	0.50
Carbomer	0.40
Acrylates/C$_{10-30}$ Alkyl Acrylates Cross-Polymer	0.25

~~Preservative~~	0.52
	~~0.10~~
Disodium EDTA	0.01
Ascorbic Acid	4.00
~~C$_{12-15}$ Alkyl Benzoate~~	~~4.00~~
Octyl Methoxycinnamate	1.00
Cetyl Alcohol	1.00
Octyl hydroxystearate	1.00
Dimethicone	0.50
Steareth-10	0.50
Tocopheryl acetate	0.10
BHT	3.50
Soymilk Powder	5.05
Sodium hydroxide 10%	1.00
Green Tea Extract	0.05
Vitamin E	0.21
Retinol	

Composition B:

Excipient	Concentration, %w/w
Deionized Water	56.80
Ethanol	20.00
Glycerin	3.00
Butylene Glycol	5.00
Propylene Glycol	3.00
Preservative	1.00
Salicylic acid	2.00
Soymilk Powder	5.00
Disodium EDTA	0.20
Hydroxypropylcellulose	1.50
Sodium Hydroxide (20%)	2.50

4. Composition A was an oil-in-water emulsion in the form of a lotion. Composition B was a gel formulation.

5. I observed the results of formulating Compositions A and B. Neither attempt was successful in obtaining a physically stable cosmetic formulation. Composition A resulted in a chunky, lumpy, physically unstable product. The phases of the composition separated and were not usable as a cosmetic. Composition B resulted in a sticky, clumpy product indicating that the product would not be usable as a topical cosmetic product.

6. The formulations of Compositions A and B contained excipients known to those of ordinary skill in the art of formulating cosmetic products at the time the invention was made. Although one of ordinary skill in the art would have expected that such compositions would have been physically stable and usable as topical cosmetics,

surprisingly, they did not result in stable, usable compositions. Rather, formulating compositions using non-denatured soy products presented exceptional challenges, including: identifying thickening agents and emulsifying agents compatible with non-denatured soy that still maintained the aesthetics required for a topical cosmetic product and developing the processes necessary to incorporate the non-denatured soy into the topical composition without damaging the soy but still maintaining physical stability.

7.   I conclude from the foregoing attempts to make physically stable compositions containing non-denatured soy products that one of ordinary skill in the art at the time the invention was made, using known cosmetic excipients, would not have necessarily been able to make physically stable, usable topical cosmetic compositions. It would, therefore, have been surprising and unexpected to obtain physically stable compositions containing non-denatured soy products.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

_____
JONATHAN D. MILLER

12/21/01
Date

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Inhibition of Human and Rat Pancreatic Proteinases by Crude and Purified Soybean Proteinase Inhibitors

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ABSTRACT   Effects of proteinase inhibitors on total proteolytic activity and trypsin and chymotrypsin activiy. in human pancreatic juice were determined separately. Purified inhibitors as well as crude extracts of raw soybeans completely inhibited trypsin and chymotrypsin activity while 40 to 50% of the total proteolytic activity remained. Inhibition experiments with 1,10-*o*-phenanthroline showed that this residual proteolytic activity was due mainly to carboxypeptidase A and B. Comparative studies with rat pancreatic enzymes demonstrated certain similarities between the corresponding enzymes from rat and man. However, differences were revealed which indicate that the rat enzymes must be used with great caution when applied as models for the human proteinases when studying effects of soybean inhibitors.   J. Nutr. *109*: 551–558, 1979.

INDEXING KEY WORDS   pancreatic proteinases · soybean inhibitors · human nutrition

In the early 1960's a program of experimental cultivation of soybeans was launched in Tanzania. The object was to produce a cheap and stable source of protein and energy which could be grown and utilized in rural areas and which was also suitable for infants and young children. Holm et al. (1) examined heat treated soybean meal from three different varieties of soya (*Glycine max*) grown in Tanzania. The results obtained in nitrogen balance experiments in rats were very different from those predicted from the amino acid patterns and this discrepancy was associated with the presence of proteinase inhibitors.

A number of inhibitors which possess different heat resistances and enzyme specificities are known to occur in soybeans. The inhibitory character of one particular variety of soybean is dependent partly on heriditary and partly on environmental factors (2, 3). The danger of variable and inadequate heat treatment is very real when the meal is produced at the village level (1). Substantial inhibitory activity

may therefore be suspected. However, the in vivo effect of proteinase inhibitors in soybean meal used as human food is not well understood.

At the present time the only report of human studies is that of Lewis and Taylor (4) who fed raw soybean meal to two nitrogen depleted adult men. Nitrogen retention was about 20% lower in the subjects fed raw soybean meal than in those fed autoclaved meal, although nitrogen digestibility was invariable. The authors concluded that these experiments indicated that soybeans are a poor source of protein unless adequately heated.

A prerequisite of a physiologically significant effect in the digestive tract brought about by proteinase inhibitors in soybeans is the formation of complexes between the proteolytic enzymes and the inhibitors, and the stabilization of these complexes suffi-

ciently to give a certain inhibition of activity. Figarella et al. (5, 6) and Laskowski et al. (7) have demonstrated complex formation between Kunitz' soybean trypsin inhibitor (SBTI) and the two human trypsins (cationic and anionic). The work of Mallory and Trawis (8) demonstrated that proteolytic activities in homogenous preparations of human cationic and anionic trypsin, chymotrypsin I and II are moderately to severely inhibited by SBTI, and by the Bowman/Birk inhibitor from soybeans (BBI) as well as by the Lima bean inhibitor (LBI). In vivo, the effects of proteinase inhibitors in soybean products on protein digestion will be an overall effect of several inhibitors on the total enzyme mixture hydrolyzing protein in the digestive tract. Accordingly, the overall effect depends both on the pattern of inhibitors present in the beans and the pattern of enzymes in the digestive tract.

Our work addresses this complex situation. To enable quantitative studies to be carried out, human pancreatic juice was employed as a model for the digestive juice in the small intestine. Extracts of raw soybeans and purified inhibitors were added to the juice and the reduction of tryptic, chymotryptic and total proteolytic activity measured. Identical experiments were carried out using pancreatic extracts from rats. The effects on the human enzymes were compared with the effects on the corresponding enzymes from rat, an experimental animal extensively used as a model system for man.

MATERIALS AND METHODS

Human pancreatic juice (HPJ). The human pancreatic juice (HPJ) was collected from the duodenum at the mouth of the pancreatic duct after intravenous administration of pancreozymin and secretin to give maximal enzyme secretion. The collection procedure was adapted from and supervised by Dr. Petersen.[2] The HPJ collected is contaminated with minor amounts of duodenal juice containing enterokinase which activates the pancreatice proenzymes (9, 10). Immediate cooling (0°), freezing in small aliquots within 1 to 2 hours (liquid N_2), and storing at −70° satisfactorily conserved the proteolytic activity. After

thawing, the juice was used at once without dilution or any other treatment. At 0° the proteolytic activity was stable for at least 12 hours. Total proteolytic activity in three collections, from three different normal subjects, varied due to variations in the water content. The activity per ml in the most dilute sample was 60% of that of the most concentrated sample. The relative activity of trypsin and chymotrypsin when measured as described below, was invariably found to be 0.4,

$$\left(\frac{\Delta T_{trypsin}}{minute}\right)\Big/\left(\frac{\Delta T_{chymotrypsin}}{minute}\right).$$

In the present experiments all HPJ used was taken from the same batch.

Rat pancreas extract (RPE). The rats used were of the Wistar strain AF/Han/MØ/Han of both sexes, 150 to 200 g.[3] The animals were given free access to food[4] and water until dissection of pancreas, at 0800, according to the method of Treadwell and Roe (11). Pancreata from 10 rats were freezeclamped, ground, and stored at −70°. The enzyme preparation (RPE) was obtained as follows: 150 mg of the powdered frozen tissue was extracted in 9 ml of buffer (0.20 M Tris-HCl, 0.05 M $CaCl_2$, pH 8.0 as measured at 30°) in the presence of 1 ml of bovine trypsin solution (0.25 mg/ml in 10^{-3} M HCl) at 4° for 24 hours in order to activate the proenzymes. This activation procedure provided maximal total proteolytic activity. (The amount of bovine trypsin used for activation is to small to be detected in any assay systems for the rat enzymes.) Proenzymes in the homogenate are not affected either by freezing or by storing at −70°. At 4° activated proteolytic enzymes in RPE are stable for at least 6 hours. The total proteolytic activity in 10 different homogenates varied only slightly. As in human pancreatic juice, the relative activity of trypsin and chymotrypsin was 0.4.

Soybean extracts (SBE). A mixture of 25 different raw soybean varieties[5] were de-

hulled and milled (80 mesh). The blended meal was used as a model of "typical" soybean meal. Solutions of proteinase inhibitors were obtained by gentle stirring of soybean meal in 0.9% NaCl (1:50 (w/v)) for 2 hours at 4° (1). The supernatants after centrifugation for 30 minutes at 10,000 × g (4°) were stored at −20°. No changes in inhibitor content were found after prolonged storage (approx. 1 year) at −20°, or after 38 hours at 4°. Before use in the assay systems all inhibitor solutions were diluted 1:2 with 0.9% NaCl.

Purified inhibitors. Kunitz soybean trypsin inhibitor (SBTI) [6] at a concentration of 0.125 mg/ml 10^{-3} M HCl and Lima bean inhibitor (LBI) [7] at 0.063 mg/ml 10^{-3} M HCl were used. LBI (M.W. ca. 9,000) was used because it is homologous to the compact, cystine-rich Bowman/Birk soybean inhibitor (BBI) which is not commercially available.

Protease and esterase assays. Total proteolytic activity was measured with casein as substrate, using a modification of the method described by Kunitz (12). The casein concentration was increased from 0.5 to 1.0% to obtain a close-to-linear relationship between enzyme activity and enzyme concentration (13). In this assay system (2.0 ml), 20 μl HPJ gave the same proteolytic activity ($OD_{280} \approx 0.7$) as 55 μl RPE. Consequently these volumes of HPJ and RPE were also employed in the following assays of individual enzymes.

The activities of trypsin (EC 3.4.21.4), chymotrypsin (EC 3.4.21.1), and carboxypeptidase A (CPA) (EC 3.4.12.2) and carboxypeptidase B (CPB) (EC 3.4.12.3) in the enzyme mixtures were measured (14, 15) using N-benzoyl-L-arginine-ethyl-ester (BAEE),[8] N-benzoyl-L-tyrosine-ethyl-ester (BTEE),[9] N-carbobenzoxy-glycyl-phenylalanine (CGP),[10] and N-hippuryl-arginine (HA),[11] respectively, as substrates. Linear relationships between the activity of each enzyme and its concentration were obtained.

Inhibition of each enzyme was measured when 10 to 200 μl of SBE, 0 to 25 μg SBTI, or 0 to 12.5 μg LBI were added to the incubation system (3.0 ml). A specific inhibitor of carboxypeptidase A and B (16), 1,10-ortho-phenanthroline,[12] was used to

characterize the enzyme composition of HPJ. Zero to ten μg were added per 20 μl HPJ, and the effects on CPA and CPB were assayed separately. In the Kunitz' procedure (13), excess 1,10-o-phenanthroline was removed by chloroform extraction (3 × 5 ml) before the spectrophotometric determination of "total proteolytic activity." Heat inactivated HPJ or RPE were obtained by heating 1 ml of the enzyme solution for 10 minutes at 100°.

The results were analyzed by two-way analysis of variance. Comparison among class means was carried out according to Snedechor and Cochran, (treatments; SBTI, LBI, and SBE, blocks; each level of inhibitor added, EMS = error of mean square[i]). The minimum level of statistical significance accepted was $P < 0.05$ (17).

RESULTS

The effects of proteinase inhibitors were studied in three series of incubation mixtures, in which total proteolytic activity, trypsin and chymotrypsin activity in human pancreatic juice (HPJ), and rat pancreatic extract (RPE) were determined separately (figs. 1A to 1C and 2A to 2C, respectively). Significant inhibition by the three inhibitor preparations was demonstrated in all assay systems (figs. 1A to 1C, 2A to 2C). The inhibition of trypsin in RPE (fig. 2B) by LBI and SBE did not differ significantly. In the remaining five essay systems, SBTI, LBI, and SBE gave different inhibition. These experiments demonstrate that proteinase inhibitors from soybeans effectively inhibit the human proteolytic enzymes. In the presence of a soybean extract (SBE) corresponding to 1.3 mg of soybean meal there was 50% inhibition of the total proteolytic activity in 20 μl of HPJ (fig. 1A), and almost complete inhibition of the trypsin and chymotrypsin activities (figs. 1B, 1C).

The total proteolytic activity of the rat

[6] Sigma type I-S no T-9003, Sigma Chemical Co., St. Louis, Missouri.
[7] Sigma type II-L no T-9878, Sigma Chemical Co., St. Louis, Missouri.
[8] Serva no 14600, Serva GmbH, Heidelberg, Germany.
[9] Serva no 14700, Serva GmbH, Heidelberg, Germany.
[10] Merck art. 2345, E. Merck, Darmstadt, Germany.
[11] Merck art. 24556, E. Merck, Darmstadt, Germany.
[12] Merck art. 7225, E. Merck, Darmstadt, Germany.
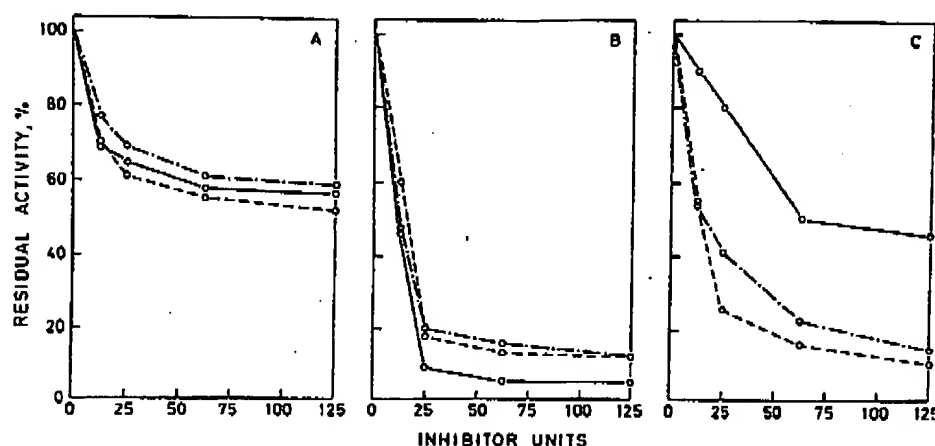
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Fig. 1 Inhibition of total proteolytic activity (A), tryptic activity (B), and chymotryptic activity (C) in human pancreatic juice (HPJ) by Kunitz' soybean trypsin inhibitor (SBTI) (————), lima bean inhibitor (LBI) (– – –), and soybean extracts (SBE) (–·–·–). Units: 10^{-3} nmole SBTI, 10^{-3} nmole LBI, 1.6 μl SBE (corresponding to 6.8 μg soybean meal extracted). A: 20 μl human pancreatic juice (HPJ) in 2.0 ml 1% casein solution (12). TCA-soluble material was determined from change in absorbance at 280 nm. EMS = 3.1. B: 20 μl HPJ in 3.0 ml BAEE solution (13). Benzoyl-arginine release was measured at 253 nm. EMS = 2.1. C: 20 μl HPJ in 3.0 ml BTEE solution (13). Benzoyl-tyrosine release was measured at 256 nm. EMS = 1.7.

enzymes (fig. 2A) was inhibited by SBE to a somewhat lesser extent than that of the human enzymes (fig. 1A). The inhibition of rat trypsin and chymotrypsin by SBE was marked (figs. 2B, 2C) and similar in magnitude to that of the corresponding human enzymes (figs. 1B, 1C). This strong inhibition of trypsin and chymotrypsin from both sources is only partly reflected in the inhibition of total proteolytic activity.

The inhibition obtained with LBI resembled that of SBE in all assay systems. The differences between the pattern of inhibition with human and rat enzymes by SBE are to a large extent reproduced by LBI. However, the amount of soybean meal required to cause a given degree of inhibition is 100 times that of crystalline LBI.

Human proteinases seem to be inhibited less by SBTI than by the same amount (moles) of LBI (figs. 1A to 1C). With rat enzymes the picture is more complicated. In RPE, trypsin was inhibited more by SBTI than chymotrypsin was. SBTI and LBI had similar effects on the tryptic activity of RPE (fig. 2B), whereas SBTI had a much weaker effect on chymotrypsin than

LBI had (fig. 2C). Differences in the pattern of inhibition of human and rat enzymes seem to be dependent on the type of inhibitor as well as the amount of inhibitor.

The relative inhibitory capacity of SBTI and LBI

$$\left(\frac{\Delta \text{ activity}}{\text{mole SBTI}}\right)\Big/\left(\frac{\Delta \text{ activity}}{\text{mole LBI}}\right)$$

towards rat trypsin differed markedly from that towards chymotrypsin. The relative inhibitory capacity of SBTI and LBI towards human trypsin and chymotrypsin was almost the same. This indicates a difference in the properties of the corresponding enzymes from man and rat.

LBI was used as a representative of the cystine-rich low molecular weight proteinase inhibitors. The inhibitory pattern of SBE appeared to be different from SBTI and similar to that of LBI. This becomes particularly clear in figures 2B and 2C. This indicates that the predominant inhibitors in SBE may be homologous to LBI and belong to the heat stable type.

When 1.6 nmole of LBI was added to the standard incubation mixtures contain-

ing HPJ, virtually no trypsin or chymotrypsin activity could be demonstrated, although approximately 40% of the total proteolytic activity was present (point ▲ in fig. 3). The importance of carboxypeptidase activity in this situation was tested in experiments using the zinc-chelating agent 1,10-o-phenanthroline. Figure 4 shows that the removal of zinc ions by this agent affects the activity of the two carboxypeptidases differently. When 1,10-o-phenanthroline was added to HPJ in which all the trypsin and chymotrypsin activity had been inhibited by 1.6 nmole of LBI, a further 25% of the total proteolytic activity was lost (fig. 3). The lowered residual activity, due to increasing amounts of 1,10-o-phenanthroline was significantly different at each level of inhibitor added.

Carboxypeptidases contribute a significant part of both the total proteolytic activity in HPJ and the activity after inhibition with LBI. The remaining 15% of proteolytic activity after inhibition with LBI and 1,10-o-phenanthroline is probably due to elastase which is not affected by the inhibitors used.

DISCUSSION

Previous findings that human proteinases were apparently little affected by soybean inhibitors prompted speculation that these inhibitors had very little relevance to human nutrition (18–20). The present work indicates that SBTI has a considerable inhibitory capacity towards human trypsin and chymotrypsin in HPJ (figs. 1A to 1C). Evidently protein digestion may be impaired by SBTI. The inhibition obtained using SBE indicates that soybean proteinase inhibitors, in general, may be of significance in human nutrition. Extracts of 70 g soybean meal were able to eliminate trypsin and chymotrypsin activity in 1 liter of HPJ. Therefore, extracts from about 100 g of raw soybean meal will be required to inhibit the trypsin and chymotrypsin secreted in 24 hours. Inhibitor patterns in soybeans are not well known. However, 25 different soybean varieties were mixed to obtain SBE, and it seems unlikely that only a few varieties should be responsible for the inhibitory character shown in all assay systems to be similar to LBI. Consequently, the low-molecular weight and heat stable inhibitor types may frequently be present.
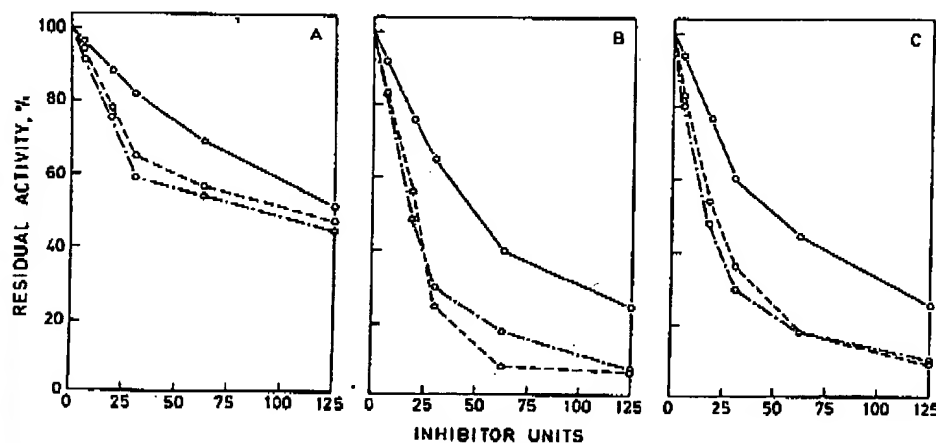

Fig. 2 Inhibition of total proteolytic activity (A), tryptic activity (B), and chymotryptic activity (C) in rat pancreas extract (RPE) by Kunitz soybean trypsin inhibitor (SBTI) (———), lima bean inhibitor (LBI) (— — —), and soybean extracts (SBI) (—·—·—). Units: 10^{-1} nmole SBTI, 10^{-3} nmole LBI, 1.8 µl SBE (corresponding to 6.8 µg soybean meal extracted). A: 55 µl rat pancreatic extract (RPE) in 2.0 ml 1% casein solution (12). TCA-soluble material was determined from change in absorbance at 280 nm. EMS = 1.2. B. 55 µl RPE in 3.0 ml BAEE solution (13). Benzoyl-arginine release was measured at 253 nm. EMS = 1.6. C: 55 µl RPE in 3.0 ml BTEE solution (13). Benzoyl-tyrosine release was measured at 256 nm. EMS = 2.4.

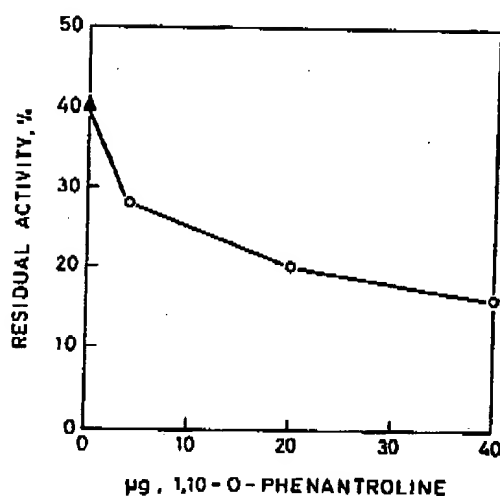556   ASHILD KROGDAHL AND HALVOR HOLM

Fig. 3 Reduction in total proteolytic activity in human pancreatic juice (HPJ) due to inhibition of carboxypeptidase A and B by 1,10-o-phenanthroline. Two milliliters "standard" casein solution containing 20 μl HPJ was made "trypsin and chymotrypsin free" by addition of 1.6 nmole LBI and residual proteolytic activity due to carboxypeptidase A and B further inhibited by 1,10-o-phenanthroline, 4 to 40 μg (15). EMS = 1.7.

The overall effects as seen in the assay of total proteolytic activity will be influenced by the inhibitor pattern and the enzyme mixture present. The inhibitors will alter the relative active enzyme concentrations and this will, in turn, alter the relative effects of the inhibitors (8). In most feeding experiments with rats raw soybeans cause enlargement of the pancreas, reduced growth, reduced protein digestibility, impaired fat absorption, lowered energy utilization, and reduced availability of amino acids, vitamins, and minerals (2). The present work shows that an extract of approximately 1 g soybean meal eliminates the trypsin and chymotrypsin activity from one rat pancreas (approx. 0.55 g) while it reduces the total proteolytic activity to about 50% (figs. 2A to 2C). A substantial part of the proteolytic activity in HPJ is shown to be due to carboxypeptidase A and B (fig. 3). In the experiments where residual proteolytic activity in LBI-treated HPJ was studied, about 25% of the total activity could be accounted for by the carboxypeptidases. The remaining activity is assumed to be due to elastase. Soybeans

are not known to contain inhibitors of the carboxypeptidases, but elastase inhibitors have been identified (21). Consequently, the endopeptidase activity in general may be reduced while the exopeptidases may be unaffected by soybean extracts.

Secretion of proteolytic enzymes from the pancreas is under humoral regulation. Pancreozymin (cholecystokinin) is the main regulating hormone. Among other stimuli, the levels of active trypsin and chymotrypsin in the duodenum are important. Low levels of active enzymes stimulate pancreozymin secretion, elevate plasma pancreozymin and hence induce proteinase secretion. Proteinase inhibitors effectively lower the level of active intestinal trypsin and chymotrypsin. Consequently, the output of proteolytic enzymes from pancreas
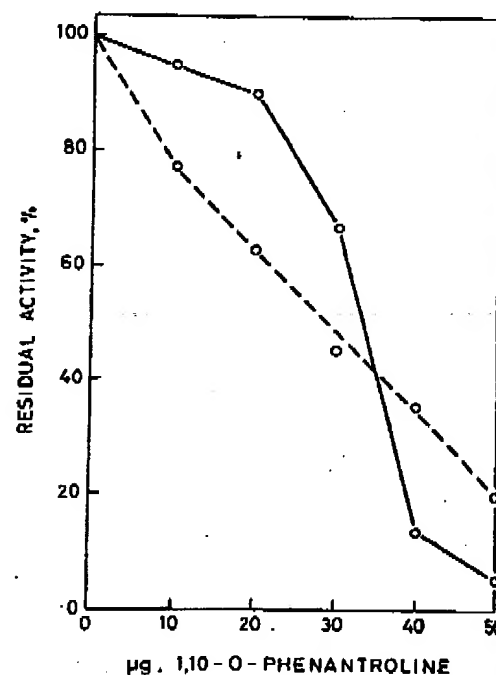

Fig. 4 Inhibition of carboxypeptidase A (CPA) and B (CPB) by 1,10-o-phenanthroline. A 3.0 ml incubation solution (15) containing 100 μl HPJ was used. CPA activity was measured as amidase activity by determining the reduced absorbance at 232 nm using N-carbobenzoxy-glycyl-phenylalanine as substrate. CPB activity was determined at 254 nm using N-hippurylarginine as substrate. CPA, EMS = 2.0. CPB, EMS = 1.8 (CPA, O——O; CPB, O---O).

increases. Continued hypersecretion from the pancreas causes enlargement of the gland with both hyperplasia and hypertrophy (22–26).

Trypsin and chymotrypsin are enzymes rich in cystine. Hypersecretion of these enzymes elevates the requirement for sulfur-containing amino acids. Sulfur-containing amino acids are usually the limiting amino acids in soybeans. Active proteinase inhibitors in soybeans therefore aggravate this deficit and bring about growth retardation (27–29). Proteinase inhibitor may cause a general delay in protein digestion. In combination with an uneven distribution of absorption mechanisms for amino acids in the small intestine, this delay may lead to impaired amino acid absorption. Absorption sites localized in the distal part of the small intestine are mainly specific for free amino acids and absorption rates vary greatly from one amino acid to another. Sites in the proximal intestine take care of small peptides supplying the organism with free amino acids at uniform rates (30). The effective utilization of amino acids requires near simultaneous absorption. Hence, proteinase inhibitors, by delaying protein digestion, may reduce amino acid utilization as well as absorption. In particular the enzyme-inhibitor complexes, · containing large amounts of sulfur-containing amino acids, are liable to escape digestion and absorption (1, 29, 31). As the content of proteinase inhibitors in soybean meal may vary appreciably (1), there is always the risk of supplying man with substantial amounts of proteinase inhibitors.

In the assay systems used, both the enzyme concentrations and the kinetics of enzyme inhibitor interaction are unknown. Comparisons between effects of SBTI, LBI, and SBE were therefore accomplished by comparing the relative effects of the inhibitors. This revealed that the inhibition of human trypsin and chymotrypsin was different from that of the rat proteinases (figs. 1B, 1C, 2B, 2C). The different inhibition obtained by LBI and SBTI points out that screening inhibitor content of different soybean varieties using rat enzymes probably ranks the varieties in a way not consistently relevant to human nutrition. Similar conditions may occur for most en-

zymes in question. It seems precarious arbitrarily to choose enzymes for testing the content of inhibitors in soybeans and, without further examination, to assume the results to be applicable to the nutrition of different animal species including man. Until now, bovine trypsin has been extensively used in proteinase inhibitor assays whether the results are intended for man, pigs, or chickens. However, nobody has demonstrated that these results in general correspond to effects on human enzymes or that they are relevant to human nutrition.

In conclusion, the inhibition of human proteinases by soybean extracts is considered relevant in human nutrition. When rat enzymes are used to predict the effect on human proteinases, great caution must be exercised. Tests should at least be carried out at several levels of inhibitors.

ACKNOWLEDGMENTS

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Effect of Dietar and Growth Re

ABSTRACT   A experiments to det ments of 1- to 21-day fat milk basal diet ning at 1 day of ag (PCV) of 21-day (ppm) of dietary The heaviest 21-day Variations were fou on body weight. Th by 21-day old chic requirement of 80 ; of 21-day old broil than a 10:1, 15:1, requirements for h same; however, the two criteria.   J. I

INDEXING KEY hematological

As early as 1929, resear that anemia in chickens co with the addition of Fe to diet (1). The need for bot animal diets has been cle: however, the relationship l Cu requirements is not cle: Copper stimulates erythrop (2–5) and more specificall tion of heme (6), wherea directly in hemoglobin fo: fore, to study the requirem(or Cu, the requirement for eral must be considered.

The Fe or Cu requiren chicks has been investigate(researchers. For maximun

Structure-Function Relationships of Proteinase Inhibitors from Soybean (Bowman-Birk) and Lima Bean

MODIFICATION BY N-ACETYLIMIDAZOLE*

Ernest Kay

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Contributions of tyrosyl residues to trypsin- and chymotrypsin-inhibitory activities in two homologous proteinase inhibitors were investigated by modifying them with N-acetylimidazole under various conditions. In Bowman-Birk soybean proteinase inhibitor, Tyr 55, immediately following the antichymotryptic site, Leu 53-Ser 54, is relatively inaccessible to N-acetylimidazole and can only be acetylated in the presence of 6 M guanidine hydrochloride but not in 8 M urea. The acetylation of Tyr 55 is accompanied by 60% loss in antichymotryptic activity. Deacetylation with hydroxylamine restores the activity to the original level. Tyr 69, located in the antitrypsin portion of the inhibitor, is exposed relatively to N-acetylimidazole and can be acetylated without denaturing agent. The acetylation of Tyr 69 parallels decrease in antitryptic activity. The inhibitor acetylated at Tyr 69 is fully active toward chymotrypsin and has 30 to 40% antitryptic activity of the native. The original level of antitryptic activity is restored upon deacetylation.

Tyr 69 of lima bean proteinase inhibitor is relatively inaccessible to N-acetylimidazole: 75% acetylation in the presence of 6 M guanidine hydrochloride and 17% without the denaturing agent. The acetylated inhibitor is fully active toward chymotrypsin but retains only 29% (acetylated without guanidine hydrochloride) and 17% (acetylated with guanidine hydrochloride) of the original antitryptic activity. Deacetylation partially restores the lost antitryptic activity in the inhibitor acetylated without the denaturing agent.

The total and irreversible loss of antitryptic activity in samples acetylated in the presence of 8 M urea or 6 M guanidine hydrochloride is attributed to the acetylation at the ϵ-amino group of Lys 26 at the trypsin-inhibitory site.

Bowman-Birk soybean proteinase inhibitor and lima bean proteinase inhibitor are "double-headed" inhibitors which inhibit trypsin and chymotrypsin at independent reactive sites (1-6). A close homology between the inhibitors can be recognized by a near-identity in their amino acid sequences; only six positions are substituted from residues 13 to 73[1] (5, 7, 8).

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[1] In order to simplify presentation, amino acid sequences of the two inhibitors are aligned and residue numbers of LBI are used for both. Therefore, the amino acid sequence of BBI starts with residue 11 (8).

BBI[2] can be cleaved into three fragments by cyanogen bromide treatment followed by pepsin digestion. One fragment, residues 11 to 37 and 67 to 77 held together by four disulfide bonds, contains the trypsin-inhibiting site Lys 26-Ser 27. It has 84% antitryptic activity of the native and no antichymotryptic activity. The second consists of residues 38 to 66, which include the chymotrypsin-inhibitory site Leu 53-Ser 54 and three disulfide bonds. The antichymotryptic fragment possesses no antitryptic activity and 16% antichymotryptic activity of the intact inhibitor. The third fragment is a tetrapeptide from the COOH terminus of the native inhibitor (9). Spectrophotometric study of BBI showed that one of two tyrosyl phenolic groups is relatively exposed and can be O-acetylated by 100- to 1500-fold molar excess of N-acetylimidazole. The other is inaccessible to the reagent even in the presence of 8 M urea, but can be acetylated in the presence of 6 M guanidine hydrochloride. Which of the 2 tyrosyl residues is exposed could not be determined (10). LBI contains only one tyrosine, Tyr 69, which is relatively inaccessible to N-acetylimidazole. A 100-fold excess of the reagent in the presence of 6 M guanidine hydrochloride elicited about 75% O-acetylation (11).

Radiation studies of BBI and LBI suggest that damage to BBI Tyr 55, adjacent to the antichymotrypsin site, leads to loss of chymotrypsin-inhibitory activity. Radiation damage to Tyr 69, located in the antitryptic fragment, has no effect on either of the antiproteinase activities in both inhibitors (11, 12).

In the present study, antiproteinase activities of BBI and LBI derivatives acetylated under various conditions have been determined and the roles of tyrosyl residues in the activities have been evaluated.

EXPERIMENTAL PROCEDURES

BBI and LBI were purified as described (10, 11). Acetylation and deacetylation of the inhibitors were performed according to reported methods (13, 14). Unreacted N-acetylimidazole was removed by dialysis against water at 4°C. Inhibitory activities of the inhibitors were measured spectroscopically, as described (12).

RESULTS

Fig. 1 presents relationship between the O-acetylation of tyrosyl side chains and the change in inhibitory activities in BBI. The treatment of the inhibitor with increasing amounts of N-acetylimidazole resulted in a progressive acetylation of tyrosyl side chains. However, only 1 of 2 tyrosyl residues in BBI could be modified by 100- to 1500-fold molar excess of the reagent. Urea (8 M) did not affect the tyrosine acetylation, as one tyrosine remained unmodified by its presence (Table I). When BBI was acetylated in the presence of 6 M guanidine

[2] The abbreviations used are: BBI, Bowman-Birk soybean proteinase inhibitor; LBI, lima bean proteinase inhibitor.

hydrochloride, however, both tyrosines were almost completely acetylated (Table I).

Antichymotrypsin activity of BBI was not affected by the acetylation of the "accessible" tyrosine, as inhibitor modified by N-acetylimidazole alone was fully active toward chymotrypsin, even after treatment with 1500-fold molar excess of the reagent (Fig. 1). Antitrypsin activity, however, paralleled the acetylation of the accessible tyrosine and decreased as the tyrosyl side chain was acetylated. At 100-fold molar excess of the reagent, antitrypsin activity was about 40% of the original; further increase did not reduce the trypsin-inhibitory activity much. At 1500-fold excess, modified BBI retained about 25% antitryptic activity of the native. The lost antitryptic activity of the modified inhibitor acetylated without denaturing agent could be recovered fully by deacetylating the O-acetylated tyrosyl side chain with hydroxylamine (Table I).

Although essentially no enhancement in tyrosine acetylation was observed in samples modified in the presence of 8 M urea, antitryptic activity was completely lost by this treatment, while antichymotryptic activity remained relatively unaffected (Table I). Deacetylation of the sample acetylated in 8 M urea restored the original antichymotryptic activity, but not the antitryptic activity.

A sample acetylated in the presence of 6 M guanidine hydrochloride and with both tyrosyl side chains O-acetylated lost all of its antitryptic activity and more than half of antichymotryptic activity (Table I). Antitryptic activity could not be regained by deacetylating O-acetyltyrosines but antichymotryptic activity was fully restored by this process.

The acetylation of LBI with a 100-fold molar excess of N-acetylimidazole induced 17% modification of tyrosine (Table I). The modified LBI lost more than two-thirds of the original antitryptic activity but retained full activity toward chymotrypsin. The deacetylation of O-acetyltyrosine by hydroxylamine realized only a partial recovery of the lost antitryptic activity.

The presence of 6 M guanidine hydrochloride during acetylation enhanced the extent of tyrosine modification to 75% from 17% (Table I). An increased loss in the antitryptic activity was also noted, but the antichymotryptic activity was unaffected by the presence of the denaturing agent. The lost antitryptic activity could not be regained by the restoration of tyrosyl side chains by hydroxylamine.

The treatment of the inhibitors with only 8 M urea, 6 M guanidine hydrochloride, or hydroxylamine without N-acetylimidazole did not have any effect on either of the antiproteinase activities.
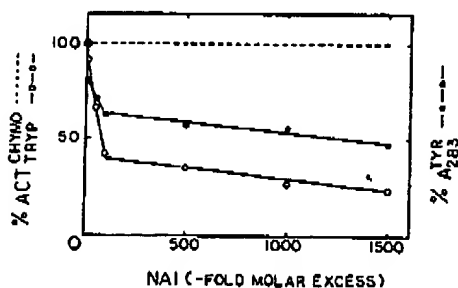
Molar excess N-acetylimidazole	Acetylated			Deacetylated	
	Per cent tyrosine acetylated	Antitrypsin	Antichymotrypsin	Antitrypsin	Antichymotrypsin
Control	0	100	100	100	100
BBI					
1500	52	41	100	100	100
1500 + 8 M urea	50	0.4	85	1.7	100
1500 + 6 M guanidine hydrochloride	97	0.4	40	1.7	100
LBI					
100	17	29	100	36	96
100 + 6 M guanidine hydrochloride	75	17	103	17	103

DISCUSSION

Of 2 tyrosyl residues in BBI, Tyr 55 is located next to antichymotryptic site Leu 53-Ser 54. The other, Tyr 69, resides in the antitryptic fragment but the proximity of this tyrosine to the trypsin-inhibitory site is unknown (9). In LBI, Tyr 69 is the sole tyrosine, as position 55 has isoleucine in the inhibitor (15).

From results of the present study, the tyrosyl residue exposed to N-acetylimidazole in BBI may be assigned to Tyr 69 in the antitryptic fragment because: (a) the loss of antitryptic activity follows closely the acetylation of the accessible tyrosine, (b) antitryptic activity can be restored to the original level by deacetylating the BBI derivative which was acetylated without denaturing agent; and (c) the acetylation of the accessible tyrosine does not have any effect on antichymotryptic activity. The other tyrosyl residue that is relatively inaccessible to N-acetylimidazole must then be Tyr 55, adjacent to the antichymotryptic active site. Additional evidence is that the acetylation of the "inaccessible" tyrosine results in 60% reduction of antichymotryptic activity which can be restored upon deacetylation.

The acetylation of Tyr 69 in BBI without denaturing agent induces 60 to 70% loss in antitryptic activity which is fully recoverable by deacetylation. The presence of 8 M urea during the acetylation does not influence the degree of tyrosine modification, but it produces a complete and irreversible loss in antitryptic activity. The presence of 8 M urea must have promoted the acetylation of other amino acid residue(s) essential to trypsin inhibitory activity. Although N-acetylimidazole is used primarily for acetylation of tyrosyl side chains, acetylation of amino groups has been demonstrated as well (13). ε-Amino groups of lysines can be modified by such a treatment (16), but only O-acetyltyrosine is deacetylated by the procedure used (13). The substitution of Lys 26 at the trypsin inhibitory site may then be suggested for the irreversible loss in antitryptic activity. Similarly, a total and irreversible loss of antitryptic activity in samples acetylated in the presence of 6 M guanidine hydrochloride may also be attributed to the irreversible acetylation of the same lysyl residue.

The results of LBI acetylation support the assignment of tyrosyl residues in BBI: The acetylation of Tyr 69 in LBI, with or without 6 M guanidine hydrochloride, has no effect on antichymotryptic activity at all while antitryptic activity is

FIG. 1. Tyrosine acetylation and antiproteinase activities of BBI acetylated with N-acetylimidazole (NAI). ●——●, per cent of tyrosine acetylation using Δε₂₈₃ = 2240 for BBI with both tyrosyl side chains O-acetylated (10); - - -, per cent change in antichymotryptic activity; O——O, per cent change in antitryptic activity (12). Acetylation was performed in 50 mM sodium borate, pH 7.5, at 24°C for 2 h (10, 14).

reduced. A part of the lost antitryptic activity in the sample modified without the denaturing agent can be recovered upon deacetylation. The irreversible acetylation of Lys 26 might also be assumed for unrecoverable loss of antitryptic activity in LBI.

A conclusion that modification of Tyr 55 results in the loss of antichymotryptic activity is in accord with a previous study using free radicals as modifying agents. In that study radiation damage to Tyr 55 was suggested to be accompanied by decline in antichymotryptic activity without affecting antitryptic activity (12). However, the modification of Tyr 69 by free radicals had no effect on either of the antiproteinase activities in both BBI and LBI (11, 12), a conclusion which seems contradictory to results of the acetylation study.

Since radiation and acetylation produce different products, the modification of the same residue in a protein by these two techniques may be expected to induce different effects on the functions. Derivatives of Br_2^- and $(CNS)_2^-$ attack on tyrosine have not been well characterized. By analogy with hydroxyl radicals, however, a major product of tyrosine degradation by these radical anions may be assumed to be 3,4-dihydroxyphenylalanine (17-19), a minor structural modification. On the other hand, acetylation introduces a relatively bulky acetyl group at the hydroxyl group. Such a bulky substitution may sterically hinder the formation of a proper enzyme-inhibitor complex.

Although the modification of Tyr 55 in BBI by either radiation or acetylation results in the loss of antichymotryptic activity, it is not an "essential" amino acid and probably does not participate directly in chymotrypsin binding. Thus it is replaced by other amino acids in inhibitors homologous to BBI without losing antichymotryptic activity, isoleucine in LBI (15) and methionine in garden bean proteinase inhibitor II (20). However, the integrity of Tyr 55 is essential to chymotrypsin-inhibitory activity in BBI.

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CONTINUING EDUCATION/REVIEWS

THE EFFECTS OF SOYBEAN TRYPSIN INHIBITORS ON THE PANCREAS OF ANIMALS AND MAN: A REVIEW

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The introduction of a new or alternative food source into the American diet often creates a myriad of questions regarding its safety and nutritional value. This is especially true when that food is composed of a complexity of components. Soybeans belong to this category. Most of the enzymes found in raw soybeans remain chemically active unless they are heated. A deleterious effect was noted on the pancreas in some species of animals fed raw soybeans. This was thought to be a result of those enzymes. For this reason, soybeans and their enzymatic components were scrutinized for any purported toxicity to man.

Inhibitors of proteolytic activity of many enzymes are found throughout the plant kingdom, particularly among the legumes. Table 1 lists the variety of plants containing protease inhibitors.

In addition to the legumes, cereal grains, grasses, potatoes and eggplant all contain protease inhibitors (1-3).

Humans have 2 types of trypsin, cationic and anionic, that are similar to mammalian trypsins in amino acid composition (4,5); however, only four disulfide bonds are possible, compared with six for other mammalian species (5).

The soybean inhibitors of trypsin (STI) in particular have received considerable attention because of their effects on animals in both growth and pancreatic activity (6-11). These effects include pancreatic hypertrophy in rats (1,7-11) and chicks (2,8,12), but not in dogs (12-15) or calves(14). Further studies in rats revealed not only hypertrophy and hyperplasia of the pancreas in rats fed continuous diets containing raw soya flour, but they also developed hyperplastic nodules and adenomas. A few of the rats developed pancreatic cancer (9). Growth inhibition and diarrhea were also seen in calves (6), rats (2), chicks (12) and mice (16).

At least five trypsin inhibitors have been isolated from soybeans (2) and soybean whey (17). According to Ikenaka et al (18), Kunitz isolated the first inhibitor in 1945; it is composed of a single polypeptide chain with approximately 200 amino acids and two disulfide bonds as shown in Figure 1.

Another trypsin inhibitor, the Bowman-Birk inhibitor, differs from the Kunitz inhibitor not only in the fact that it is a more potent

Fig 1. Kunitz Inhibitor

inhibitor of trypsin, but it also displays powerful inhibitory activity toward chymotrypsin. This inhibitor contains 17 disulfide bonds which account for its high resistance to severe treatment with heat, acid, alkali, pepsin or papain. The lima bean (Phaseolus lunatus) and other legumes also contain similar resistant trypsin inhibitors (2).

MECHANISM OF ACTION OF INHIBITORS

Substantial evidence indicates that the feeding of raw soybeans and purified soy trypsin inhibitors accelerates protein synthesis in the pancreas and stimulates hypersecretion of pancreatic enzymes (amylase, lipase and trypsin) into the intestinal tract (1,7,8). The secretory response of the pancreas to dietary trypsin inhibitors is an indirect response that is initiated in the intestine and not in the blood. In rats, pancreatic enzyme secretion is suppressed by negative feedback inhibition resulting from the presence of trypsin and chymotrypsin in the intestinal tract. The trypsin inhibitors increase pancreatic enzyme secretion by forming inactive trypsin-trypsin inhibitor complexes and therefore decrease the suppression exerted by free trypsin (1,11). Feedback inhibition occurs in humans and pigs, but not in dogs (1). The presence of protein and trypsin inhibitors in the duodenum results in a release of cholecystokinin (CCK) from the binding sites in the mucosa. CCK also has trypsin inhibiting activity and can cause pancreatic hypertrophy and inhibition of rat growth (1), as shown in Figure 2.

Table 1. Distribution of Protease Inhibitors in Plants

Botanical Name	Common Name	Part of Plant
Arachis hypogeae	Peanut, ground nut	Seed skin
Artocarpus integrifolia	Jack fruit	Seed
Avena sativa	Oats	Seed
Beta vulgaris	Beet, beetroot	Root
Brassica rapa	Turnip	Root
Cajanus cajan	Red gram	Seed
Cajanus indicus	Pigeon peas	Seed
Canvalia ensiformis	Jack bean	Seed
Ceratonia siligua	Carob bean	Seed
Cercis canadensis	Redbud tree	Seed
Chamaecrista fasiculata	Partridge pea	Seed
Cicer arietinum	Bengal gram, chick pea, garbanzo	Seed
Colocasia esculenta	Taro	Root
Cyanopsis psoraloides	Guar bean	Seed
Dolichos lablab	Field bean, hyacinth bean	All parts
Faba vulgaris	Double bean	All parts
Fagopyrum esculentum	Buckwheat	Seed
Gleditsia tricanthos	Honey locust	Seed
Glycine max	Soybean	Seed
Glymnocladus dioica	Kentucky coffee bean	Seed
Hordeum vulgare	Barley	Seed
Ipomoea bacata	Sweet potato, yam	Root and leaves
Lactuca sativa	Lettuce	Seed
Lens esculenta	Lentil	Seed
Lespedeza stipulacea	Lespedeza	Seed
Mendicago sativa	Alfalfa, lucerne	Leaf
Mucana deeringianum	Florida velvet bean	Seed
Oryza sativa	Rice	Seed
Phaseolus aureus	Green gram, mung bean	Leaves
Phaseolus coccineus	Scarlet runner bean	Seed
Phaseolus vulgaris	Garden bean	Seed
Phaseolus lunatis	Lima bean	Seed

A casein diet is also a stimulant of pancreatic secretion in the rats by binding to trypsin during digestion with the result that feedback inhibition is decreased (1).

INHIBITOR SPECIES SPECIFICITY

Endogenous inhibitors to trypsin have been found to bovine trypsin, porcine trypsin and human trypsin. Human pancreatic secretory trypsin inhibitor effectively inhibits human
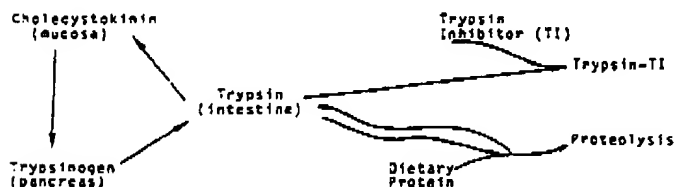
Fig 2. Regulation of trypsin secretion

Vet Hum Toxicol 24, 1 Feb 1982

cationic and anionic trypsin, bovine trypsin and porcine trypsin. The cationic human trypsin is not inhibited by bovine or porcine trypsin inhibitors (4,19).

Trypsin inhibitors can discriminate between bovine and human trypsin (20). Soybean trypsin inhibitor can inhibit human trypsin 2 (anionic), 100% in a 1:1 molar ratio as it inhibits bovine trypsin; however, human trypsin 1 (cationic) is inhibited only 40% at a 1:1 molar ratio (4). The reaction of human trypsin with soybean trypsin inhibitor is shown in Figure 3.

Inhibitors known to possess a susceptible bond of type Arg-X (4) are among the poorest inhibitors of human trypsin, especially trypsin 1. The soybean trypsin inhibitor is classified in this group. The primary structure of the reactive site of modified STI is
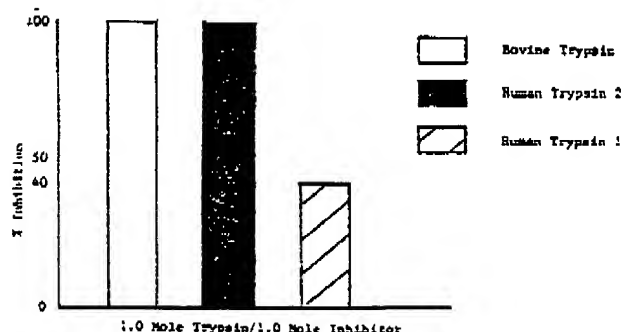
Fig 3.   Inhibition of bovine and human trypsin with
soybean trypsin inhibitor

shown below, the residues labeled according
to the notation of SCHECHTER and BERGER (21):

P4 P3 P2 P1   :   P1 P2 P3 P4
Pro Ser Tyr Arg OH : H Ile Arg Phe He

The weak inhibition of human trypsin 1 by
STI has never been described for the trypsin
of any other mammalian species. In addition,
the trypsin that fails to be inhibited by STI
represents the major part of the potential
trypsin activity of the whole juice (about
2/3) (4). Most authors found weak and poorly
reproducible inhibition of human trypsin by
STI (1,4,5,20,22). This is due to lesser
stabilization of the trypsin-inhibitor com-
plex (23).

TRYPSIN INHIBITORS AND CANCER

Unlike the effects of STI on man, rats de-
veloped pancreatic hypertrophy (1,2,7,9-11)
which may act as a promoter for potential
pancreatic carcinogens. Raw soya flour alone
fed to rats led to the development of hyper-
plastic nodules, adenomas and, in some cases,
cancer (9). Combining two known carcinogens,
azaserine and di(2-hydroxypropyl)nitrosamine,
with raw soya flour diets augmented their
carinogenicity in the pancreas of rats (24,25).

Mice fed diets containing raw soybeans,
however, showed a delay in the appearance of
tumors initiated with nitroquinoline-oxide
(N O) by 45 days and a decrease in the number
of tumors by 50% after 200 days (26).

SUMMARY

Human trypsin is more resistant to inhibi-
tion than is the trypsin of other mammalian
species. The effect on human trypsin of soy-
bean trypsin inhibitor in soy protein does
not appear to be a potential hazard to man.
Therefore, the elimination of STI does not
seem to be necessary for humans. In animal
diets, however, pancreatic toxicity must be
considered whenever soybean protein is uti-
lized. Soybeans should be treated to increase
their nutritional benefits and decrease any
animal health risks (27-29). This will in-
sure healthy control subjects in laboratory
situations and avoid misinterpretation of
pathologic data. The treatment suggested is
heat (2,18,25,30-32) since heat will destroy
most of the soybean trypsin inhibitors. Ad-

ditional supplementation is required following
heat treatment for amino acids (33,34) such
as methionine, valine, and threonine; for
choline (2,14,35); and for the minerals zinc
(36) and calcium (11,34). Excessive heat
must be avoided since it will decrease the
nutritional value of soybean protein and
increase lysinoalanine, a nephrotoxic sub-
stance (12). Finally, the use of STI as a
promotor in the study of potential pancreatic
carcinogens may prove beneficial for cancer
research (24,25) and might be considered in
the future.

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THE SOLDIER had just arrived at a camp near his home after three years overseas and was very eager to be reunited with his wife. But try as he would, he could only get a two-hour leave. After a six-hour absence, he came back to camp. "Why are you four hours AWOL?" barked the sergeant. "Well," replied the soldier, "when I got home I found my wife in the tub, and it took me four hours to dry out my uniform."